Rhizobium taibaishanense sp. nov., isolated from a root nodule of *Kummerowia striata*

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During a study of the diversity and phylogeny of rhizobia in the root nodules of *Kummerowia striata* grown in north-western China, four strains were classified in the genus *Rhizobium* on the basis of their 16S rRNA gene sequences. The 16S rRNA gene sequences of three of these strains were identical and that of the other strain, which was the only one isolated in Yangling, differed from the others by just 1 bp. The 16S rRNA gene sequences of the four strains showed a mean similarity of 99.3% with the most closely related, recognized species, *Rhizobium vitis*. The corresponding *recA* and *glnA* gene sequences showed similarities with established species of *Rhizobium* of less than 86.5% and less than 89.6%, respectively. These low similarities indicated that the four strains represented a novel species of the genus *Rhizobium*. The strains were also found to be distinguishable from the closest related, established species (*R. vitis*) by rep-PCR DNA fingerprinting, analysis of cellular fatty acid profiles and from the results of a series of phenotypic tests. The level of DNA–DNA relatedness between the representative strain CCNWSX 0483^T and *Rhizobium vitis* IAM 14140^T was only 40.13%. Therefore, a novel species, *Rhizobium taibaishanense* sp. nov., is proposed, with strain CCNWSX 0483^T (=ACCC 14971^T=HAMB1 3214^T) as the type strain. In nodulation and pathogenicity tests, none of the four strains of *Rhizobium taibaishanense* sp. nov. was able to induce any nodule or tumour formation on plants. As no amplicons were detected when DNA from the strains was run in PCR with primers for the detection of *nodA*, *nifH* and *virC* gene sequences, the strains probably do not carry *sym* or *vir* genes.

The species once assigned to the genus *Agrobacterium* Conn 1942 are Gram-reaction-negative soil bacteria that were placed in the family *Rhizobiaceae* of the class *Proteobacteria* on the basis of their rRNA gene characteristics (Kerstens *et al.*, 1973; Woese *et al.*, 1984). Most of these micro-organisms are plant pathogens that cause the formation of crown galls or hairy roots on various plant species, and their classification was once mainly based on their pathogenic properties. According to Young *et al.* (2001), the 16S rRNA gene sequences of species of the genus *Agrobacterium* are so similar to those of species of the genus *Rhizobium* that all *Agrobacterium* species should be transferred into the genus *Rhizobium*. Although this

transfer has largely been accepted, it remains a subject of controversy and debate (Farrand *et al.*, 2003).

Kummerowia is a genus of flowering plants belonging to the subfamily Faboideae of the family Leguminosae. Species of the genus *Kummerowia* are drought-enduring and can be used as fodder for cattle, as green manure and/or to prevent soil erosion (Allen & Allen, 1981). In China, some *Kummerowia* species are also valued as medicines and are used for the treatment of traumatic injuries with blood stasis, dysentery, icteric hepatitis, sores, carbuncles, furuncles and abscesses (New Medicine College of Jiangsu, 1977). Species of the genus *Kummerowia* are also being considered for the treatment of menopausal dysfunction, since they are a source of oestrogen (Yoo *et al.*, 2005).

Cui *et al.* (2010) recently isolated 53 rhizobial strains from the root nodules of *Kummerowia striata* grown in north-western China and explored the strains' phenotypes and genetics. Analysis of their 16S rRNA gene sequences indicated that four of the strains (CCNWSX 0483^T,

The GenBank accession numbers for the 16S rRNA, *recA* and *glnA* gene sequences of strain CCNWSX 0483^T are HM776997, HM777002 and HQ846353, respectively.

A supplementary table and two supplementary figures are available with the online version of this paper.

CCNWSX 0487, CCNWSX 0481 and CCNWSX 0457) were closely related to *Rhizobium vitis*, with a mean sequence similarity of 99.3%. In the present study, to verify their taxonomic position, these four strains were further characterized by sequence analyses of the *recA* and *glnA* housekeeping genes, repetitive element PCR fingerprinting (rep-PCR), DNA–DNA hybridization, the determination of cellular fatty acid profiles and numerical taxonomy. In addition, the existence of *sym* and *vir* genes in the four strains was checked in PCR-based assays and plant inoculation tests. All of the strains were grown on yeast-mannitol agar (YMA; Vincent, 1970) at either 28 °C or, for temporary storage, 4 °C.

Total genomic DNA, isolated and purified as described by Moulin *et al.* (2004), was used as PCR template. The universal primers P1/P6 (Tan *et al.*, 1997) were used to amplify the almost-complete 16S rRNA gene sequence (Wang *et al.*, 1998) whereas partial *recA* and *glnA* gene sequences were determined as described by Gaunt *et al.* (2001) and Turner & Young (2000), respectively. All of the sequences were compared with those found by BLAST searching in the NCBI sequence database (Altschul *et al.*, 1990). Sequences were aligned using the CLUSTAL W program (Thompson *et al.*, 1994) before the distances between the sequences were calculated using the maximum

composite likelihood method (Tamura *et al.*, 2004), the Kimura two-parameter method (Kimura, 1983) and the methods of Jukes & Cantor (1969) and Tajima & Nei (1984). Phylogenetic trees were constructed using the MEGA 4.1 package (Kumar *et al.*, 2008) and the neighbour-joining (Saitou & Nei, 1987), minimum-evolution (Rzhetsky & Nei, 1993) and maximum-parsimony methods (Swofford, 1993). The robustness of the tree branches was estimated from bootstrap analysis, with 1000 replications. There were no significant topological differences between the phylogenetic trees created using the three treeing methods. The trees shown (Figs 1 and 2) were inferred by the neighbour-joining method after distance analysis of the aligned sequences according to the maximum composite likelihood model. In the trees for the 16S rRNA gene sequences, strains CCNWSX 0483^T, CCNWSX 0487, CCNWSX 0481 and CCNWSX 0457 formed one tight group within the genus *Rhizobium* (Fig. 1). The observed 16S rRNA gene sequences of three of these strains were, in fact, identical, and that of the other strain (CCNWSX 0457, isolated in Yangling) differed from these by just 1 bp. In terms of their 16S rRNA gene sequences, strains CCNWSX 0483^T, CCNWSX 0487, CCNWSX 0481 and CCNWSX 0457 appeared to be most closely related to *Rhizobium vitis*, with a mean similarity of 99.3%. Phylogenetic analyses of the partial *recA* and *glnA* sequences supported the conclusions

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences, showing the phylogenetic position of *Rhizobium taibaishanense* sp. nov. within the genus *Rhizobium*. Bootstrap values ($\geq 50\%$) based on 1000 resamplings are shown at branch nodes. Bar, 0.01 substitution per nucleotide position.

Fig. 2. The results of rep-PCR analyses showing the relationship between *R. vitis* and *Rhizobium taibaishanense* sp. nov. Clustering analysis based on the combined ERIC- and BOX-PCR fingerprints was performed using the UPGMA method.

drawn from the 16S rRNA gene sequences (Supplementary Figs S1 and S2, available in IJSEM Online). The observed *recA* and *glnA* gene sequences of strains CCNWSX 0483^T, CCNWSX 0487 and CCNWSX 0481 were identical and showed 97.8 % (*recA*) and 98.5 % (*glnA*) similarities with the corresponding sequences of strain CCNWSX 0457. For *recA*, strain CCNWSX 0483^T showed the highest sequence similarities with *Rhizobium rosettiformans* W3^T (86.5 %) and *Rhizobium rhizogenes* NCPPB 2991^T (82.8 %). For *glnA*, the strains of recognized species that appeared most closely related to CCNWSX 0483^T were *R. rhizogenes* LMG 150^T (88.9 %), *Ensifer adhaerens* LMG 20216^T (88.8 %) and *R. vitis* S4 (88.5 %). The low similarities found between the *recA* and *glnA* gene sequences of strains CCNWSX 0483^T, CCNWSX 0487, CCNWSX 0481 and CCNWSX 0457 and the corresponding sequences of any recognized species indicated that the four Chinese strains represent a novel species of the genus *Rhizobium*.

Rep-PCR is a useful method for differentiating closely related rhizobia and can be accurate to strain level (Gao *et al.*, 2001; Kawaguchi *et al.*, 2008; Laguerre *et al.*, 2003; Menna *et al.*, 2009). In the present study, the primers ERIC1R (5'-AT-GTAAGCTCCTGGGGATTAC-3') and ERIC2 (5'-AAGT-AAGTGACTGGGGTGAGCG-3') were used for ERIC-PCR (Versalovic *et al.*, 1991) while BOXAIR (5'-CTACGGCA-AGGCGACGCTGACG-3') was used as the primer for BOX-PCR (Versalovic *et al.*, 1994). In these rep-PCR, each type of DNA fragment with a different electrophoretic mobility was scored as either 1 (if present) or 0 (if absent). The BioNumerics program (Applied Mathematics), with the UPGMA algorithm and the Jaccard coefficient (Sneath & Sokal, 1973), were used for the cluster analysis. The results of the rep-PCR analysis confirmed that strains CCNWSX 0483^T, CCNWSX 0487, CCNWSX 0481 and CCNWSX 0457 were each different from *Rhizobium vitis* IAM 14140^T. The

rep-PCR results for strains CCNWSX 0483^T, CCNWSX 0487 and CCNWSX 0481 were identical, while those for strain CCNWSX 0457 indicated a similarity level of 66 % with the other three strains (Fig. 2), demonstrating that the four strains were not all clones of one strain.

DNA–DNA hybridization is a useful method for identifying novel bacterial species, especially when the 16S rRNA gene sequence of the novel species has a high level of similarity with that of an established species (Graham *et al.*, 1991). Total DNA was therefore isolated from strains CCNWSX 0483^T, CCNWSX 0487, CCNWSX 0481 and CCNWSX 0457 and purified (Marmur, 1961). The DNA G + C content was determined by the thermal denaturation method of Marmur & Doty (1962), with *Escherichia coli* K-12 used as the standard. The G + C content of the four Chinese strains (58.0–59.2 mol%) fell within the range reported for the genus *Rhizobium* (Jordan, 1984). DNA–DNA relatedness was then estimated by following the thermal melting protocol of De Ley *et al.* (1970). Although the level of DNA–DNA relatedness among the four Chinese strains ranged from 89.04 % to 96.30 %, that between the representative Chinese strain (CCNWSX 0483^T) and the reference strain of *R. vitis* (IAM 14140^T) was only 40.13 % (Table 1), again indicating that strains CCNWSX 0483^T, CCNWSX 0487, CCNWSX 0481 and CCNWSX 0457 represent a novel species.

Cellular fatty acid profiles have been used to distinguish between species of *Rhizobium* and those of related genera (Tighe *et al.*, 2000). To measure the fatty acid composition of strain CCNWSX 0483^T, the strain was incubated at 28 °C for 48 h on TY medium (Jarvis *et al.*, 1996). The methods subsequently used for harvesting, saponification, methylation and extraction of the cellular fatty acids followed version 3.0 of the Sherlock Microbial Identification System

Table 1. Strains used in this study and their DNA–DNA relatedness

Strain	Geographical origin	Host plant	DNA G + C (mol%)	DNA–DNA relatedness with CCNWSX 0483 ^T (%)
<i>Rhizobium taibaishanense</i> sp. nov. CCNWSX 0483 ^T	Taibaishan Mountains, Shaanxi, China	<i>Kummerowia striata</i>	58.0	100
CCNWSX 0487	Taibaishan Mountains, Shaanxi, China	<i>Kummerowia striata</i>	58.0	96.30
CCNWSX 0481	Taibaishan Mountains, Shaanxi, China	<i>Kummerowia striata</i>	58.2	92.80
CCNWSX 0457	Yangling, Shaanxi, China	<i>Kummerowia striata</i>	59.2	89.04
<i>Rhizobium vitis</i> IAM 14140 ^T	Australia	<i>Vitis</i> sp.	59.8	40.13

(MIDI, 1999). A Hewlett Packard 6890 gas chromatograph equipped with an Ultra2 capillary column (25 m × 0.2 mm) and a flame-ionization detector was then used to separate the fatty acid methyl esters. For this chromatography, hydrogen was used as the carrier gas, nitrogen was the 'makeup' gas, and air was used to support the flame. Peak integration and identification were performed using the MIDI software package (Sherlock TSBA40 method and TSBA40 library; MIDI Inc.). The cellular fatty acids of strain CCNWSX 0483^T were identified as C_{18:1ω7c} (65.02 %), C_{16:0} (6.47 %), C_{16:0} 3-OH (3.22 %), iso-C_{17:0} 3-OH (1.63 %), C_{18:1ω7c} 11-methyl (2.31 %), C_{18:0} 3-OH (1.09 %), C_{18:1} 2-OH (1.50 %), C_{19:0} cyclo ω8c (6.18 %) and C_{19:0} 10-methyl (1.66 %), with three summed features (Supplementary Table S1), indicating that the strain should be assigned to the genus *Rhizobium* (Tighe *et al.*, 2000). Strain CCNWSX 0483^T differed from recognized species of the genus *Rhizobium* in its lack of detectable C_{18:0} and C_{20:3ω6,9,12c}, its higher levels of iso-C_{17:0} 3-OH, and its possession of C_{18:0} 3-OH.

The phenotypic features of strains CCNWSX 0483^T, CCNWSX 0487, CCNWSX 0481 and CCNWSX 0457 were also compared with those of *R. vitis* IAM 14140^T, using the methods described by Gao *et al.* (1994). Twenty-four different carbon sources and 15 types of nitrogen source were tested. Resistance to chloramphenicol, lincomycin, azithromycin, kanamycin, amikacin sulfate, streptomycin sulfate, ampicillin and benzylpenicillin sodium, each at concentrations of 300, 100, 50 and 5 µg ml⁻¹ in YMA, was also investigated. The temperature range for growth was determined by incubating cultures on YMA at 4, 40 and 60 °C. The effect of pH on growth was explored using YMA at final pH values of 4.0, 5.0, 9.0, 10.0, 11.0 and 12.0 and the method of Moffett & Colwell (1968). Salt tolerance was studied in YMA containing 1–6 % (w/v) NaCl. Dye and chemical resistance was investigated with methyl orange, methyl red, methylene blue, neutral red, Congo red, malachite green, bromothymol blue, sodium deoxycholate and NaNO₂, each at concentrations of 1 % and 2 % (w/v) in YMA. Reactions in litmus milk, alkali production from malonic acid, utilization of citrate and production of

3-ketolactose were examined by the methods of Moore *et al.* (1988). Indole production, reactions in the methyl red and Voges–Proskauer tests, hydrolysis of starch, gelatin and Tween 80, catalase, urease and oxidase activities, reduction of nitrate and nitrite, and hydrogen sulphide production from cysteine were also determined, as described by Smibert & Krieg (1994). Distinctive phenotypic characteristics of strains CCNWSX 0483^T, CCNWSX 0487, CCNWSX 0481 and CCNWSX 0457 and the type strains of the phylogenetically closest species are shown in Table 2. Strains CCNWSX 0483^T, CCNWSX 0487, CCNWSX 0481 and CCNWSX 0457 each differed from *R. vitis* in their ability to utilize β-naphthyl acetate, hydrolyse gelatin, peptonize and reduce litmus milk and grow at 40 °C, and in their inability to utilize (–)-L-sorbose and citrate, produce alkali from litmus milk, and grow in the presence of 2 % (w/v) NaCl.

Attempts were made to amplify intragenic fragments of any *nodA*, *nifH* and *virC* genes in strains CCNWSX 0483^T, CCNWSX 0487, CCNWSX 0481 and CCNWSX 0457, to see if the strains harboured the *sym* and *vir* genes that can induce the formation of root nodules and plant tumours, respectively. Amplification of the *nodA* fragment (Haukka *et al.*, 1998) was based on the primers *nodA*-F1 (5'-TGC-RGTGGARDCTRYGCTGGGAAA-3') and *nodA*-R2 (5'-G-GNCCGTCRTCAASGTCARGTA-3'). The primers *nifH*40F (5'-GGNATCGGCAAGTCSACSAC-3') and *nifH*817R (5'-TCRAMCAGCATGTCCTCSAGCTC-3') were used for the amplification of the *nifH* fragment (Vinuesa *et al.*, 2005), whereas amplification of the *virC* fragment (Sawada *et al.*, 1995) was based on VCF (5'-ATCATTTGTAGCGACT-3') and VCR (5'-AGCTCAAACCTGCTTC-3'). Although the expected amplicons were produced when the three different PCR were run with the DNA of *R. leguminosarum* USDA 2370^T (*nodA* and *nifH* amplifications) or *R. vitis* IAM 14140^T (*virC* amplifications) used as positive controls, no amplicons were detected when the template DNA came from strains CCNWSX 0483^T, CCNWSX 0487, CCNWSX 0481 or CCNWSX 0457.

To assess their symbiotic potential, strains CCNWSX 0483^T, CCNWSX 0487, CCNWSX 0481, CCNWSX 0457

Table 2. Major characteristics that distinguish *Rhizobium taibaishanense* sp. nov. from the closest recognized species, *R. vitis*

Taxa: 1, *R. vitis* IAM 14140^T; 2, *R. taibaishanense* sp. nov. CCNWSX 0487; 3, *R. taibaishanense* sp. nov. CCNWSX 0483^T; 4, *R. taibaishanense* sp. nov. CCNWSX 0481; 5, *R. taibaishanense* sp. nov. CCNWSX 0457. +, Positive; –, negative.

Characteristic	1	2	3	4	5
Substrates used as sole carbon source:					
(–)-L-Sorbose	+	–	–	–	–
β-Naphthyl acetate	–	+	+	+	+
Sodium succinate	+	–	+	+	+
Substrates used as sole nitrogen source:					
L-Alanine	+	–	+	+	+
L-Cysteine	+	–	+	+	–
DL-α-Aminopropionic acid	+	–	+	+	–
L-Tyrosine	+	–	+	+	+
Antibiotic resistance:					
Benzylpenicillin sodium (50 µg ml ^{–1})	+	+	+	+	–
Benzylpenicillin sodium (100 µg ml ^{–1})	+	+	+	+	–
Benzylpenicillin sodium (300 µg ml ^{–1})	+	+	+	–	–
Ampicillin (50 µg ml ^{–1})	+	–	+	+	–
Streptomycin sulfate (50 µg ml ^{–1})	+	–	+	+	–
Streptomycin sulfate (100 µg ml ^{–1})	–	–	+	–	–
Streptomycin sulfate (300 µg ml ^{–1})	–	–	+	–	–
Amikacin sulfate (5 µg ml ^{–1})	+	–	+	+	+
Tolerance to:					
Malachite green (1 %)	+	–	+	+	–
NaCl (1 %)	+	+	+	+	–
NaCl (2 %)	+	–	–	–	–
NaCl (3 %)	+	–	–	–	–
Growth at:					
pH 4	+	+	–	+	+
40 °C	–	+	+	+	+
Hydrolysis of Tween 80	–	–	–	–	+
Gelatin liquefaction	–	+	+	+	+
Citrate utilization	–	+	+	+	+
Litmus milk alkali production	+	–	–	–	–
Litmus milk peptonization	–	+	+	+	+
Reduction of litmus milk	–	+	+	+	+

and, as a positive control, the symbiotic strain *Ensifer kummerowiae* CCBAU 71714^T (which was isolated from *K. stipulacea*) were each inoculated onto seedlings grown in pots filled with vermiculite that had been moistened with N-free plant nutrient solution (Vincent, 1970). The inoculated plants were *K. striata* (i.e. the host of origin of strains CCNWSX 0483^T, CCNWSX 0487, CCNWSX 0481 and CCNWSX 0457) and two species of leguminous plant that often coexist with *K. striata* in north-western China: *Melilotus officinalis* and *Medicago sativa*. The inoculated plants were allowed to grow for 1 month before being checked for signs of root nodulation. Although all three plant species inoculated with the positive control developed pink nodules, no nodules were detected on any of the other plants.

The ability of strains CCNWSX 0483^T, CCNWSX 0487, CCNWSX 0481, CCNWSX 0457 and, as a positive control, the tumour-inducing *R. vitis* IAM 14140^T to produce crown galls was investigated by inoculating 4-week-old tomato seedlings and 3-week-old sunflower seedlings (Moore *et al.*, 2001). After the plants were left to grow for 1 month, only those inoculated with the positive control were found to have developed crown galls.

The results of the present study indicate that strains CCNWSX 0483^T, CCNWSX 0487, CCNWSX 0481 and CCNWSX 0457 represent a novel species related to, but distinct from, *R. vitis*, for which we propose the name *Rhizobium taibaishanense* sp. nov.

Description of *Rhizobium taibaishanense* sp. nov.

Rhizobium taibaishanense (tai.bai.shan.en'se. N.L. neut. adj. *taibaishanense* of or belonging to the Taibaishan Mountains in the Shaanxi province of China, where the bacterium was isolated).

The morphology of the cells and colonies is as described for the genus *Rhizobium*. Produces acid on YMA and uses sodium pyruvate, sodium malonate, D-xylose, galactose, D-fructose, sodium propionate, L-arabinose, raffinose, D-ribose, rhamnose, sodium hippurate, β-naphthyl acetate, α-naphthalene acetic acid, mannose, maltose, trisodium citrate, sodium acetate, glucose, sucrose, lactose, sodium tartrate and inositol as sole carbon source; some strains can also use sodium succinate but none of those tested can use (–)-L-sorbose or starch. L-Cystine, L-methionine, L-lactamine, L-glutamic acid, L-asparagine, L-threonine, L-leucine, L-adenine, L-arginine and L-phenylalanine are used as sole nitrogen sources; some strains can also use L-cysteine, DL-α-aminopropionic acid, L-tyrosine and L-alanine but none of those tested can utilize DL-histidine. Grows on YMA medium at 4 and 40 °C, at pH 12.0, and with 1 % NaCl (except strain CCNWSX 0457), 0.2 % methyl orange, 0.2 % methyl red, 0.2 % neutral red, 0.2 % Congo red, 0.2 % bromothymol blue, 0.2 % sodium deoxycholate, 0.2 % NaNO₂, 300 µg chloramphenicol ml^{–1}, or 300 µg lincomycin ml^{–1}. However, it is sensitive to azithromycin at 5 µg ml^{–1}, kanamycin at 50 µg ml^{–1} and amikacin sulfate at 50 µg ml^{–1}. Does not produce 3-ketolactose or H₂S, but does produce alkali from citric and malonic acids. Produces catalase and oxidase, reduces nitrate and nitrite, hydrolyses gelatin, and peptonizes and reduce litmus milk. Gives negative results in methyl red, Voges–Proskauer and indole tests. Of the tested strains, only CCNWSX 0457 can utilize Tween 80.

The type strain, CCNWSX 0483^T (=ACCC 14971^T=HAMBI 3214^T), was isolated from a root nodule of *Kummerowia striata* grown in the Taibaishan Mountains, in the Shaanxi province of China. The DNA G+C content of the type strain is 58.0 mol%.

Acknowledgements

This work was supported by the 973 Project of China (2010CB126502), National Science Foundation of China (30970003, 30900215 and 30630054), International Science and Technology Cooperation Program of China (2010DFA91930), and the Key Laboratory of Protection and Utilization of Biological Resources in Tarim Basin, Xinjiang Production and Construction Group (BRZD1002). We are grateful to Dr Kristina Lindström and Professor Ruibo Jiang for the deposition of the type strain in their culture collections.

References

- Allen, O. N. & Allen, E. K. (1981). *The Leguminosae: A Source Book of Characteristics, Uses and Nodulation*. Madison, WI: The University of Wisconsin Press.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Conn, H. J. (1942). Validity of the genus *Alcaligenes*. *J Bacteriol* **44**, 353–360.
- Cui, G. L., Xie, R. M., Yue, J. J., Liang, J. Q., Duan, X. D., Fan, L. M. & Wei, G. H. (2010). Diversity and phylogeny of rhizobia isolated from *Kummerowia* in northwest of China. *J Agric Biotechnol* **18**, 867–875 (in Chinese).
- De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.
- Farrand, S. K., Van Berkum, P. B. & Oger, P. (2003). *Agrobacterium* is a definable genus of the family *Rhizobiaceae*. *Int J Syst Evol Microbiol* **53**, 1681–1687.
- Gao, J. L., Sun, J. G., Li, Y., Wang, E. T. & Chen, W. X. (1994). Numerical taxonomy and DNA relatedness of tropical rhizobia isolated from Hainan province, China. *Int J Syst Bacteriol* **44**, 151–158.
- Gao, J. L., Terefework, Z., Chen, W. X. & Lindström, K. (2001). Genetic diversity of rhizobia isolated from *Astragalus adsurgens* growing in different geographical regions of China. *J Biotechnol* **91**, 155–168.
- Gaunt, M. W., Turner, S. L., Rigottier-Gois, L., Lloyd-Macgilp, S. A. & Young, J. P. W. (2001). Phylogenies of *atpD* and *recA* support the small subunit rRNA-based classification of rhizobia. *Int J Syst Evol Microbiol* **51**, 2037–2048.
- Graham, P. H., Sadowsky, M. J., Keyser, H. H., Barnet, Y. M., Bradley, R. S., Cooper, J. E., De Ley, D. J., Jarvis, B. D. W., Roslycky, E. B. & other authors (1991). Proposed minimal standards for the description of new genera and species of root- and stem-nodulating bacteria. *Int J Syst Bacteriol* **41**, 582–587.
- Haukka, K., Lindström, K. & Young, J. P. W. (1998). Three phylogenetic groups of *nodA* and *nifH* genes in *Sinorhizobium* and *Mesorhizobium* isolates from leguminous trees growing in Africa and Latin America. *Appl Environ Microbiol* **64**, 419–426.
- Jarvis, B. D. W., Sivakumaran, S., Tighe, S. W. & Gillis, M. (1996). Identification of *Agrobacterium* and *Rhizobium* species based on cellular fatty acid composition. *Plant Soil* **184**, 143–158.
- Jordan, D. C. (1984). Genus I. *Rhizobium* Frank 1889, 338^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 235–242. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
- Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, pp. 21–132. Edited by H. N. Munro. London: Academic Press.
- Kawaguchi, A., Sawada, H. & Ichinose, Y. (2008). Phylogenetic and serological analyses reveal genetic diversity of *Agrobacterium vitis* strains in Japan. *Plant Pathol* **57**, 747–753.
- Kerstens, K., De Ley, J., Sneath, P. H. A. & Sackin, M. (1973). Numerical taxonomic analysis of *Agrobacterium*. *J Gen Microbiol* **78**, 227–239.
- Kimura, M. (1983). *The Neutral Theory of Molecular Evolution*. Cambridge: Cambridge University Press.
- Kumar, S., Nei, M., Dudley, J. & Tamura, K. (2008). MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. *Brief Bioinform* **9**, 299–306.
- Laguerre, G., Louvrier, P., Allard, M. R. & Amarger, N. (2003). Compatibility of rhizobial genotypes within natural populations of *Rhizobium leguminosarum* biovar *viciae* for nodulation of host legumes. *Appl Environ Microbiol* **69**, 2276–2283.
- Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J Mol Biol* **3**, 208–218.
- Marmur, J. & Doty, P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. *J Mol Biol* **5**, 109–118.
- Menna, P., Pereira, A. A., Bangel, E. V. & Hungria, M. (2009). rep-PCR of tropical rhizobia for strain fingerprinting, biodiversity appraisal and as a taxonomic and phylogenetic tool. *Symbiosis* **48**, 120–130.
- MIDI (1999). *Sherlock Microbial Identification System Operating Manual, version 3*. Newark, DE: MIDI Inc.
- Moffett, M. L. & Colwell, R. R. (1968). Adansonian analysis of the *Rhizobiaceae*. *J Gen Microbiol* **51**, 245–266.
- Moore, L. W., Kado, C. I. & Bouzar, H. (1988). *Agrobacterium*. In *Laboratory Guide for Identification of Plant Pathogenic Bacteria*, 2nd edn, pp. 16–36. Edited by N. W. Schaad. St Paul, MN: American Phytopathology Society.
- Moore, L. W., Bouzar, H. & Burr, T. (2001). *Agrobacterium*. In *Laboratory Guide for Identification of Plant Pathogenic Bacteria*, 3rd edn, pp. 17–35. Edited by N. W. Schaad, J. B. Jones & W. Chun. St Paul, MN: American Phytopathology Society.
- Moulin, L., Béna, G., Boivin-Masson, C. & Stępkowski, T. (2004). Phylogenetic analyses of symbiotic nodulation genes support vertical and lateral gene co-transfer within the *Bradyrhizobium* genus. *Mol Phylogenet Evol* **30**, 720–732.
- New Medicine College of Jiangsu (1977). *Dictionary of Chinese Traditional Medicine*, Part 1. Shanghai: Shanghai People Publishing Company.
- Rzhetsky, A. & Nei, M. (1993). Theoretical foundation of the minimum-evolution method of phylogenetic inference. *Mol Biol Evol* **10**, 1073–1095.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sawada, H., Ieki, H. & Matsuda, I. (1995). PCR detection of Ti and Ri plasmids from phytopathogenic *Agrobacterium* strains. *Appl Environ Microbiol* **61**, 828–831.
- Smibert, R. M. & Krieg, N. R. (1994). Phenotypic characterization. In *Methods for General and Molecular Bacteriology*, pp. 607–654. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.
- Sneath, P. H. A. & Sokal, R. B. (1973). *Numerical Taxonomy. The Principles and Practice of Numerical Classification*. San Francisco: W. H. Freeman and Co.
- Swofford, D. L. (1993). PAUP: Phylogenetic analysis using parsimony, version 3.1.1. Champaign, IL: Illinois Natural History Survey.
- Tajima, F. & Nei, M. (1984). Estimation of evolutionary distance between nucleotide sequences. *Mol Biol Evol* **1**, 269–285.
- Tamura, K., Nei, M. & Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proc Natl Acad Sci U S A* **101**, 11030–11035.

- Tan, Z. Y., Xu, X. D., Wang, E. T., Gao, J. L., Martínez-Romero, E. & Chen, W. X. (1997). Phylogenetic and genetic relationships of *Mesorhizobium tianshanense* and related rhizobia. *Int J Syst Bacteriol* **47**, 874–879.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Tighe, S. W., de Lajudie, P., Dipietro, K., Lindström, K., Nick, G. & Jarvis, B. D. W. (2000). Analysis of cellular fatty acids and phenotypic relationships of *Agrobacterium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* species using the Sherlock Microbial Identification System. *Int J Syst Evol Microbiol* **50**, 787–801.
- Turner, S. L. & Young, J. P. W. (2000). The glutamine synthetases of rhizobia: phylogenetics and evolutionary implications. *Mol Biol Evol* **17**, 309–319.
- Versalovic, J., Koeuth, T. & Lupski, J. R. (1991). Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res* **19**, 6823–6831.
- Versalovic, J., Schneider, M., de Bruijn, F. J. & Lupski, J. R. (1994). Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods Mol Cell Biol* **5**, 25–40.
- Vincent, J. M. (1970). *A Manual for the Practical Study of Root Nodule Bacteria*. Oxford: Blackwell Scientific.
- Vinuesa, P., Silva, C., Lorite, M. J., Izaguirre-Mayoral, M. L., Bedmar, E. J. & Martínez-Romero, E. (2005). Molecular systematics of rhizobia based on maximum likelihood and Bayesian phylogenies inferred from *rrs*, *atpD*, *recA* and *nifH* sequences, and their use in the classification of *Sesbania* microsymbionts from Venezuelan wetlands. *Syst Appl Microbiol* **28**, 702–716.
- Wang, E. T., van Berkum, P., Beyene, D., Sui, X. H., Dorado, O., Chen, W. X. & Martínez-Romero, E. (1998). *Rhizobium huautlense* sp. nov., a symbiont of *Sesbania herbacea* that has a close phylogenetic relationship with *Rhizobium galegae*. *Int J Syst Bacteriol* **48**, 687–699.
- Woese, C. R., Stackebrandt, E., Weisburg, W. G., Paster, B. J., Madigan, M. T., Fowler, V. J., Hahn, C. M., Blanz, P., Gupta, R. & other authors (1984). The phylogeny of purple bacteria: the alpha subdivision. *Syst Appl Microbiol* **5**, 315–326.
- Yoo, H. H., Kim, T., Ahn, S., Kim, Y. J., Kim, H. Y., Piao, X. L. & Park, J. H. (2005). Evaluation of the estrogenic activity of Leguminosae plants. *Biol Pharm Bull* **28**, 538–540.
- Young, J. M., Kuykendall, L. D., Martínez-Romero, E., Kerr, A. & Sawada, H. (2001). A revision of *Rhizobium* Frank 1889, with an emended description of the genus, and the inclusion of all species of *Agrobacterium* Conn 1942 and *Allorhizobium undicola* de Lajudie et al. 1998 as new combinations: *Rhizobium radiobacter*, *R. rhizogenes*, *R. rubi*, *R. undicola* and *R. vitis*. *Int J Syst Evol Microbiol* **51**, 89–103.